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ORIGINAL ARTICLE

Cloning, expression, purification and functional analysis of a specific multi-epitope protein from multi drug resistance *Acinetobacter baumannii*

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ABSTRACT

Background and Objectives: Immunization is a promising strategy to combat against the life-threatening infections by Multi Drug Resistance *Acinetobacter baumannii*. In this study, we directed to design and evaluate the efficacy of a recombinant multi-epitope protein against this pathogen.
Materials and Methods: Epitopes prediction was performed for candidate proteins OmpA and BAM complex (BamA, BamB, BamC, BamD, BamE) from *A. baumannii*, using immune-informatics tools with high affinity for the human HLA alleles. After expression and purification of the recombinant protein, its functional activity was confirmed by interaction with positive sera.
Results: Cloning and expression of the desired multi-epitopes protein were verified. Circular Dichroism study showed the secondary structure and proper refolding of the recombinant protein was achieved and matched with computational prediction. There was a significant interaction between designed protein with antibodies presented in ICU patients' and staff's sera.
Conclusion: The interaction of the recombinant protein with patients' sera antibodies suggests that it may be a promising determinant protein for immunization against of MDR *A. baumannii.*

Keywords: Multi drug resistant; *Acinetobacter baumannii*; Recombinant multi-epitope protein (rMEP); OmpA; BAM complex

INTRODUCTION

Acinetobacter baumannii is an opportunistic nosocomial Gram-negative bacilli that causes a various ranges of infections in hospitalized patients in ICU (intensive care units) and injured war soldiers (1). Currently, antibiotics are the only treatment for infections caused by this pathogen. Resistant *A. baumannii* strains to all available antibiotics even Carbapenems, has been reported worldwide (2).

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However, colistin is the last treatment resort for MDR *A. baumannii* but its use has been limited because of drug side effects such as neurotoxicity and nephrotoxicity. Since infections by multi- or pan-drug resistant *A. baumannii* has created high mortality rates, prolonged hospitalization and financial cost of treatment (1, 3, 4), achieving an immunogenic protein can play an important role in vaccine development to prevent multi drug resistant infections (5). According to the previous studies, vaccination with whole cell pathogen, outer membrane complex Ata (6, 7), FilF (8), capsule components (9), Bap (10) and outer membrane vesicles (11) have been created immune responses against *A. baumannii*. Ideally, an appropriate vaccine candidate antigen is conserved in clinical strains that play an important role in pathogenesis of microorganism. Outer membrane proteins (Omps) act as efficient protective antigens because they are capable of stimulating host humoral immune system and providing prevention against bacterial challenges (12-14). It has an important role in adherence and interaction with eukaryotic cells, biofilm formation (15, 16), host cell apoptosis (17), stimulation of immune responses, and biogenesis of outer membrane vesicles (16). In Gram negative bacteria β-Barrel Assembly Machine (BAM) is a protein complex (18) that assemble folding and insertion of β-barrel proteins into the bacterial outer membrane. Because of crucial role of complex BAM in outer membrane protein assembling, it is highly conserved among the Gram negative bacteria. The BAM protein is composed of BamA, BamB, BamC, BamD and BamE proteins (19, 20). Due to the lack of a licensed vaccine against *A. baumannii*, different proteins and peptides might be tested to achieve an appropriate and effective vaccine candidate. According to the recent studies regarding the *A. baumannii*, new combination antigens OmpK/Omp22 (21), FilF/NucAb (22), OMV/Bap, and OmpA/Bap (23) have produced the partial to complete protection against bacteria in mice.

The sequencing of microbial genomes has been provided the potential antigens of each pathogen (24). Recently, a new method based on immuneinformatics for prediction of antigenic epitopes from numerous proteins is established. The advantages of the epitope-based proteins are high specific, no pathogenicity due to failure to return to the active state in the host, being financially and temporally cost-effective, reducing adverse effects in comparison with killed and attenuated form of vaccines (25). Therefore, we designed our study based on the novel recombinant multi-epitopes protein called rMEP consists of the immunogenic epitopes fragments from OmpA, BamA, BamB, BamC, BamD and BamE determinant antigens of *A. baumannii*. Subsequently, reaction of the recombinant multi-epitopes protein with sera from ICU staff and recovering patients with *A. baumannii* infections was evaluated by ELISA, and Western blotting.

MATERIALS AND METHODS

Study design and ethics statement. The current research was carried out in two parts. In the first part, we designed and evaluated the cloning, expression and purification of the recombinant multi-epitope protein, also we have analyzed the secondary structures and appropriate folding of the purified rMEP protein. In the second part, we confirmed reaction of the rMEP protein with sera of ICU staff and recovering patients with *A. baumannii* using ELISA and Western blotting test.

Required permissions and consent was taken from university hospitals management as well ICU staff and patients (Ethics Committee number IR.SBMU.RETECH.REC.1399.195).

Bacterial strains and plasmid vector. MDR *A. baumannii* clinical isolate strain, was prepared from the microbial collection of Microbiology Department, Shahid Beheshti University of Medical Sciences. The prepared MDR *A. baumannii* was found to be non-susceptible to antibiotics includes cefotaxime, ceftazidime, meropenem, imipenem, jentamicin, amikacin, ciprofloxacin, cefepime, piperacillin, piperacillin/tazobactam, co-trimoxazole, tetracycline and only it is susceptible to colistin. In addition, the strain harboured $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, and $bla_{\text{OXA-24-like}}$ genes (26). *E. coli* strain Top10 was used for the cloning of construct and *E. coli* strain BL21 (DE3) (Stratagene, La Jolla, CA) as well pET26b vector were used for the expression of the recombinant protein.

Construction of the recombinant protein: *In silico* design and evaluation of the rMEP protein construct. The full-length proteins sequences of OmpA (WP_000777878.1), BamA (WP_001983709.1), BamB (WP_001072121.1), BamC (WP_001297320.1),

BamD (WP_000056813.1) and BamE (WP_001170994.1) antigens of *A. baumannii*, the highly MDR AYE strain were retrieved from UniProt (https://www.uniprot.org/proteomes/UP000002446) in FASTA format. Multiple sequences alignment performed between the sequences from different strains using Clustal W2 software (https://www.ebi.ac.uk/Tools/msa/clustalo/) to identify conserved fragments in *A. baumannii* sub species reported sequences from different areas. We applied online servers to predict B-cell and T-Cell – MHC I and II binding epitopes. Prediction of B-cell epitopes performed using IEDB (http://tools.iedb.org), ABCpred (http://crdd.osdd.net/raghava/abcpred/). To identify T-cell epitopes several servers such as IEDB, ProPred (http://crdd.osdd.net/raghava/propred1/) with parameters for human HLA allele class II (HLA-DRB1*11, 13, 15, 03, 04) employed. The screened B-cell and T-cell epitopes were joined using GGGGS linkers to design protein construct that named rMEP.

Computed physical and chemical parameters of the rMEP. Physical and chemical characters including molecular weight, theoretical isoelectric point (pI), extinction coefficient, half-life, instability index, grand average of hydropathy (GRAVY) and total number of positive and negative residues were computed using the ProtParam tool (http://expasy.org/tools/protparam.html) . Antigenicity, solubility and flexibility of the rMEP was considered in epitopes prediction by IEDB server.

Prediction, and validation of the rMEP protein structure properties. Prediction of secondary, tertiary structure and modeling of rMEP were achieved using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) online web server.

The geometry quality of designed protein was validated using Ramachandran analysis at (https://servicesn.mbi.ucla.edu/PROCHECK/) and Z-score of ProSA server at (https://prosa.services.came.sbg.ac.at/prosa.php) and confirmed via Ram- achandran and Z plots.

Codon usage and mRNA structure optimization. The final amino acid sequence was reverse translated into nucleotides and codons were subsequently considered based on *E. coli* BL21 codon usage by JCAT server (http://www.JCAT.com/). At the end, 3D folding of mRNA, was predicted by mfold web server (http://unafold.rna.albany.edu/q=mfold). Efficiency of translation was analyzed based on structural stability and Gibbs free energy (ΔG). Finally, rMEP gene was chemically synthesized by Generay CO. (Shanghai, China).

Gene cloning, expression, and purification of the rMEP. The amplification of the synthetic gene was performed using primers (forward primer 5′ ggatccatgctgggctatacctttcagg3′ and revers primer 5′ aagcttcgcgctcacgcggccgttatc 3′). The BamHI and HindIII restriction sites were added to the 5’ and 3’ ends of the sequence using PCR. The PCR product was cloned into pET26b expression vector after double digestion. The vector transformed chemically into competent *E. coli* BL21 (DE3). Positive colonies were confirmed by restriction enzyme digestion and PCR. The transformed bacteria were grown in LB broth and screening was carried out by resistance to kanamycin antibiotic (30 μg/ml). For induction of the protein expression we must use 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Scientific, USA) after reaching OD at 600 nm to 0.4- 0.6. Then, the cells were harvested by centrifugation and treated with lysis buffer (100 mM NaH2PO4, 10 mM Tris–HCL, 8M urea, pH 8.0) and followed by sonication on ice in the presence of PMSF (1 mM) as a protease inhibitor. The Bacterial lysate was centrifuged and the supernatant was purified by a Ni-NTA affinity column (Qiagen, Hilden, Germany) based on the standard protocol. The purified protein was refolded to the active form by stepwise urea removal using Amicon-30KDa filter (washing the purified protein with buffers without urea) and concentration of protein was estimated by Bradford assay. Finally, the purified protein was visualized by 12% SDS-PAGE (Coomassie brilliant blue staining) and identified by Western blotting. In order to Western blot analysis, after SDS-PAGE, the separated protein was transferred on nitrocellulose membrane using a semidry transfer system (Bio-Rad, USA). After blocking of the membrane with 3% skim milk in PBST (PBS 1% + Tween20), it was washed with PBST. The blocked membrane was incubated with anti-His tag antibody alkaline phosphatase (ALP) conjugated (Sigma-Aldrich, USA) then membrane was washed in PBST and developed with 3, 3′ diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, USA).

Circular dichroism spectrophotometry. To identify the secondary structures and proper folding of the purified protein we used far-UV circular dichroism (CD) spectroscopy, JASCO J-810 Spector (Japan, Tokyo). The samples were prepared with a concentration of 0.5 mg/ml, in PBS buffer. The samples and controls in the quartz cuvette with path length 1 mm were scanned in the far-UV region 180- 240 and speed of 200 nm/min at room temperature. Data quantification was carried out by the CAPITO web server (https://data.nmr.uni-jena.de/capito/index.php). The results were compared with the *in silico* prediction data.

Serum sampling. 20 blood samples were obtained from university hospitals ICU staff, 20 samples from patients with a nosocomial infection as well as 20 samples from healthy people were prepared.

Human sera were analyzed using an optimized indirect ELISA for the presence of specific total antibodies against *A. baumannii* pathogen. The cut-off level was obtained using the mean optical density (OD) of triplicate wells corresponding to the last serum dilution of the healthy group. Sensitivity and specificity of the cut-off was determined.

Confirmation of the collected sera by *A. baumannii* ELISA. The total antibody level of all serum samples against *A. baumannii* lysate was measured using indirect Enzyme-Linked Immunosorbent Assay (ELISA). The 96-well micro plate (SPL Life Sciences, Korea) was coated by 0.5 µg of the pooled clinical isolated *A. baumannii* and diluted sera (1:50 to 1:6400) were identified by the ELISA assay according to our previous study method (27). *E. coli* and PBS used as negative controls. The cut-off value was determined by 20 negative serum samples ELISA results. The patients' sera were analyzed based on the cut-off value. They were used for additional assays.

Recombinant multi-epitope protein in interaction with antibodies: Pre-absorption of the serum samples. To avoid background reactivity and improve ELISA specificity, a pre-absorption step was performed on serum samples. For this purpose, sera were incubated with *E. coli* (Top10) lysate at 37°C, 2 hours, and overnight at 4°C. The sera were centrifuged 10 min, 1000 rpm at 37°C and the supernatant was collected and dilute with galactose 0.2 M for using in ELISA assay (28).

Reaction of the rMPE with human serum antibodies. Reaction of positive sera to recombinant multi- epitope protein was determined by ELISA. Briefly, the purified protein (0.5 µg/ml) was coated in 96-well micro plate and incubated overnight at 4°C. After washing and blocking steps, the plate incubated with serial dilutions (1:10 to 1:6400) of each serum sample from ICU staff, patients and control groups. Then the plate washed and incubated with anti- human HRP conjugated antibody (Abcam, UK). After washing, TMB (Tetra Methyl Benzidine) was used as substrate and reaction was stopped by 2N H2SO4. The absorbance of each well was measured at 450 nm using an ELISA reader. *E. coli* and PBS used as controls.

Western blot analysis. Western blot analysis was performed using rMEP protein. After SDS-PAGE, the separated protein was transferred on to nitrocellulose membrane using a semidry transfer system (Bio-Rad, USA). After blocking of the membrane with 3% skim milk in PBST (PBS 1% + Tween20), it was washed with PBST. The blocked membrane was incubated with the pool of positive sera in equal proportions, as a first antibody, at a dilution of 1:1000. Anti-human IgG that conjugated with horseradish peroxidase (HRP) (Sigma-Aldrich, USA) was used as a secondary antibody that developed with NBT-BCIP (Sigma-Aldrich, USA).

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com and Origin(Pro), Version Number 2019b. Origin Lab Corporation, Northampton, MA, USA. Data was analyzed by student t-test, One-way analysis of variance (ANOVA), Tukey HSD post hoc and bivalent Pearson correlation tests. Results were reported as mean ± standard deviation (SD). P value < 0.05 were considered statistically significant.

RESULTS

Design and analysis of the multi-epitopes protein. The sequences of selected OmpA, BamA, BamB, BamC, BamD and BamE proteins of *A. baumannii* were retrieved from the Uniprot. Based on the results of multiple sequence alignments between several genes belong to *A. baumannii* different strains in

data bank, we selected the homologous sequences for epitope mapping. Based on the results of the B-cell epitopes prediction, a total of seven linear epitopes of the proteins were selected with fixed length 20 aa and best score were selected (Table 1). The T-cell epitopes prediction was considered with fixed length 15 aa and 100% conservancy level among the *A. baumannii* strains. Seven low- percentile –rank (percentile rank <1.5) epitopes were selected for good binding peptides (Table 1). Linker consisting GGGGS was introduced between epitopes for efficient and flexible separation.

The designed amino acid sequence (rMEP) was presented as followed:

MLGYTFQDSQHKVAEKLSEYPGGGGSMLVAAPLAAADASRLGGGGSALVSAMAAVQQAYAADDFVVGGGGSNAVKQLLLRKYGNAGGGGGSDANGDSQYDTEKGECKVPEEGGGGSKSGIKYQLSALPLGGGGGGSPVVLGSDLIVGDLDGVLHLIGGGGSVAAYRGKQRLWEKKVGGGGSYKITMLALSLGVASAFVGCSGGGGSKGEVAAYRGKQRLWEGGGGSSGKEETEETTNTPVEAPKSEGGGGSQNFVDLIRRFPSSQYGGGGSKLVLTLFVTSLLAGCSIFGVGGGGSFQQVRFLLGSPTVTDGGGGSSSSEAPSASPADNEADDAAQGGGGSKSATDAANAAQNRGGGGSYKITMLALSLGVASAFVGCSGGGSYQNFVDLIRRFPSSQGGGGSTTAFGAAVVGGDNGRVSA

Underlined fragments were linker sequences.

Physicochemical properties and antigenicity of the rMEP. ProtParam results showed that the designed protein contained 421 amino acids and the average molecular weight and theoretical pI of it were 44.5kDa and 6.08 respectively. In addition, ProtParam results estimated half-life *in vitro* and *in vivo* (*Escherichia coli*) 30 and 10 hours, respectively. The instability index (II) was computed to be 37.25. This classifies the multi-epitope as stable protein. The aliphatic and GRAVY indexes of poly epitope were calculated 83.11 and -0.178, respectively. In addition, rMEP should had appropriate properties in term of antigenicity, allergenicity, solubility, and toxicity. The overall antigenicity of the rMEP was esteemed 0.52.

Computational properties of the secondary and tertiary structures of the rMEP. The results of secondary structures prediction indicated that multi-epitope consisted of 41.93% alpha helix, 45.06 % random coil and 13.01% extended strand without any beta turn in secondary structures (Fig. 1). Tertiary structure prediction of protein was performed by I-TASSER server (Fig. 1). Confidence score (C-score), template modeling score (TM-score) and root-mean-square deviation (RMSD) for 3D models were -2.66, 0.41 ± 0.14, and 13.3 ± 4.1Å, respectively. The stereo chemical quality of above I-TASSER models were analyzed by Ramachandran plot (29). The results revealed that 54.9%, 32.5%, and 12.6% of residues in multi-epitope protein were located in the favorite, allowed and outlier regions, respectively. Also the Z-score of this model obtained from ProSA web was -1.5 that was in the range of native conformations (Fig. 1).

Table 1. Predicted and selected linear B-cell and T-cell – MHC I and II Binding epitopes of OmpA, Bam A, Bam B, Bam C, Bam D, Bam E of *A. baumannii* via IEDB server.

Protein	B-cell Epitope	IEDB score*	T-cell Epitope	IEDB score**
OmpA	LGYTFQDSQHKVAEKLSEYP	1.133	MLVAAPLAAADASRL	0.54
BamA	ALVSAMAAVQQAYAADDFVV	1.104	NAVKQLLLRKYGNAG	0.19
	DANGDSQYDTEKGECKVPEE	1.035	KSGIKYQLSALPLGG	0.7
BamB	PVVLGSDLIVGDLDGVLHLI	1.124	VAAYRGKQRLWEKKV	1.14
	TTAFGAAVVGGDNGRVSA	1.520		
BamC	YKITMLALSLGVASAFVGCS	1.099	KGEVAAYRGKQRLWE	1.48
	SSSEAPSASPADNEADDAAQ	1.302	KSATDAANAAQNR	1.21
BamD	SGKEETEETTNTPVEAPKSE	1.092	QNFVDLIRRFPSSQY	0.13
	YKITMLALSLGVASAFVGCS	1.230	YQNFVDLIRRFPSSQ	0.78
BamE	KLVLTLFVTSLLAGCSIFGV	1.136	FQQVRFLLGSPTVTD	1.48

* IEDB B-cell epitope scores show antigenicity, accessibility, flexibility, hydrophilicity of prediction epitopes. High score is better. ** IEDB T-cell epitope scores show IC50 values for peptides binding to specific MHC molecules. Low score is reliable.

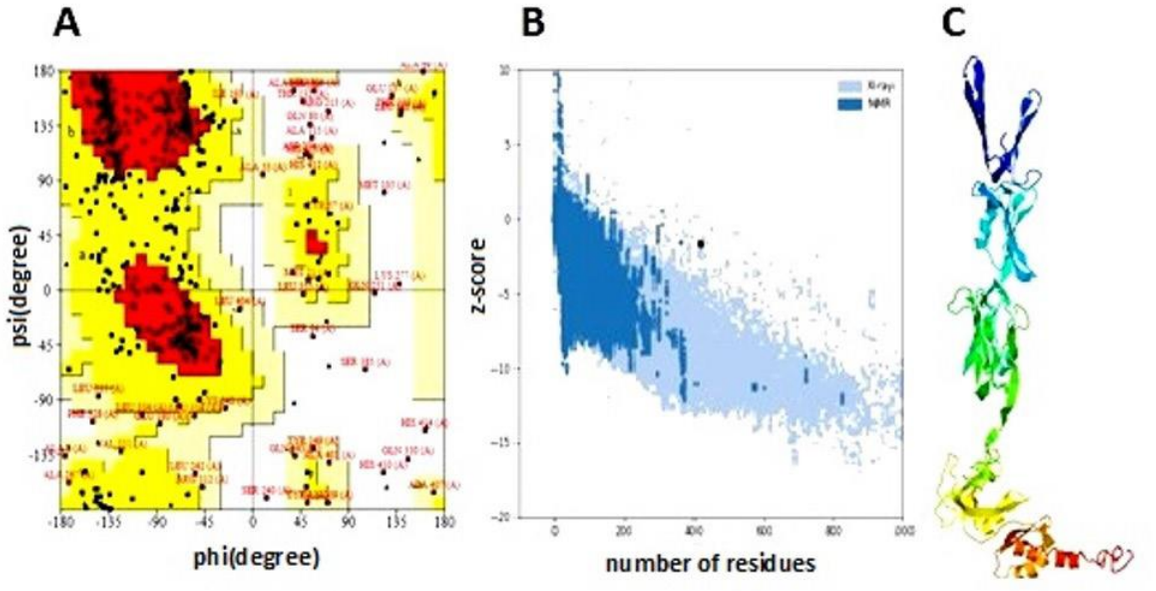

Fig. 1. rMEP 3D structure analysis. A) Ramachandran for validation geometry quality of multi-epitope protein structure. B) The Z plot of this model was -1.5 that was in the range of native conformations. C) Prediction of the 3D structure of multi-epitope protein using I-TASSER server with C-score 2.66, Estimated TM-score 0.41 ± 0.14 and Estimated RMSD 13.3 ± 4.1Å

Validation of the mRNA structure. After reverse translation of the rMEP, prediction of mRNA structure was carried out by mfold server. The results of mfold showed that ΔG of the best predicted structure was -353.5 kcal/mol and the 5′ end of the sequence has not any stable hairpin or pseudoknot. Finally, the multi epitope DNA sequence with LC521880 accession number was released in the DDBJ data bank.

Cloning, expression and confirmationof the rMEP. The synthetic designed gene was cloned successfully into pET26b as an expression vector. The existence of fragment in recombinant vector, were confirmed by restriction analysis (Fig. 2). Recombinant protein was successfully expressed in *E. coli* BL21 (DE3). Optimum expression was obtained with 1 mM IPTG and incubation time of 5 hours at 37°C. Recombinant protein was mostly expressed as inclusion body and purified under denaturing condition by Ni-NTA affinity chromatography. The purified protein was analyzed by SDS-PAGE and Western blotting (Fig. 3A). The result showed a specific band with a molecular weight of approximately 44.5 KDa. Recombinant expression of the rMEP was performed by Western blot analysis using anti His tag antibody (Fig. 3B).

Circular dichroism spectrophotometry. To monitor conformational changes in purified rMEP sec-

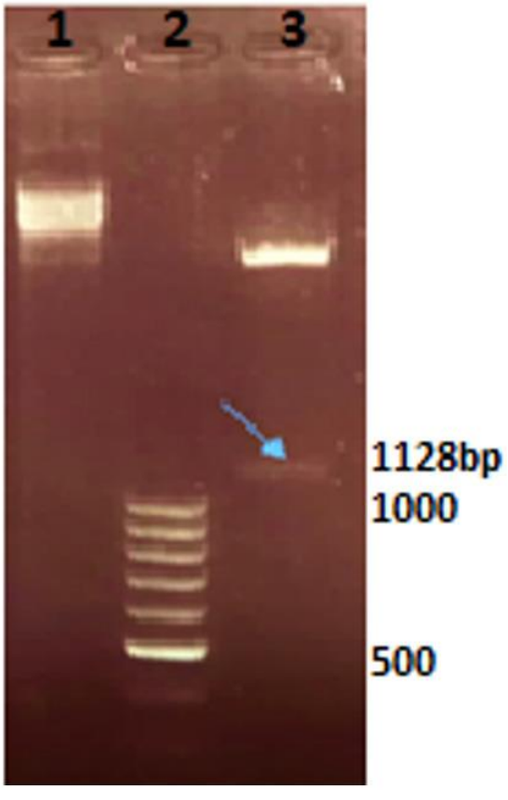

Fig. 2. Double digestion of the recombinant plasmid using *BamHI* and *HandIII* restriction enzymes. lane 1, Undigested pET26b-rMEP; lane 2, 100bp DNA Ladder; lane 3, Double digestion of the pET26b- rMEP sequence, insertion size is ~1128bp.

ondary structures, far-UV CD analysis was performed. The molar ellipticity [θ] (deg cm² mol -1) at 208 nm, 222 nm and at the ratio of 208/222 showed α-helix content. Slight changes were observed in the secondary structures of the protein compared to in silico analysis (Table 2). The data of the CD spectra of recombinant protein in the far UV range was shown in Table 2 and confirmed the rMEP secondary structures were in agreement with its in silico prediction. It seems that part of the random coil predicted structure of the rMEP protein has been converted to beta-strand structure *in vitro* due to the presence of ions in the buffer or other technical changes.

Confirmation of the collected positive sera. Human positive sera were analyzed using an indirect ELISA with extracted *A. baumannii* lysate. The cut-off level was obtained (OD= 0.22) using the mean optical density (OD) of triplicate wells corresponding to the last serum dilution of the healthy group. Sensitivity and specificity of the cut-off was determined 65% and 90% respectively. The results revealed that 4 of the 20 patient's sera were negative and that the remaining samples were positive. ICU staff's sera assessment has shown that 18 of the 20 samples were positive and 2 samples were negative. All healthy group' sera analysis did not show any reaction in ELISA. The data reported the mean of OD450 ± SD with P> 0.001 (Fig. 3).

Evaluation of rMEP in interaction with positive sera antibodies: Western blot analysis. Western blot analysis of purified rMEP using pooled positive sera as primary antibody were developed with secondary anti-human IgG HRP-conjugated that visualized with NBT-BCIP (Nitroblue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate). The results showed favorite band with desired molecular weight ~ 45.5 KDa (Fig. 3C).

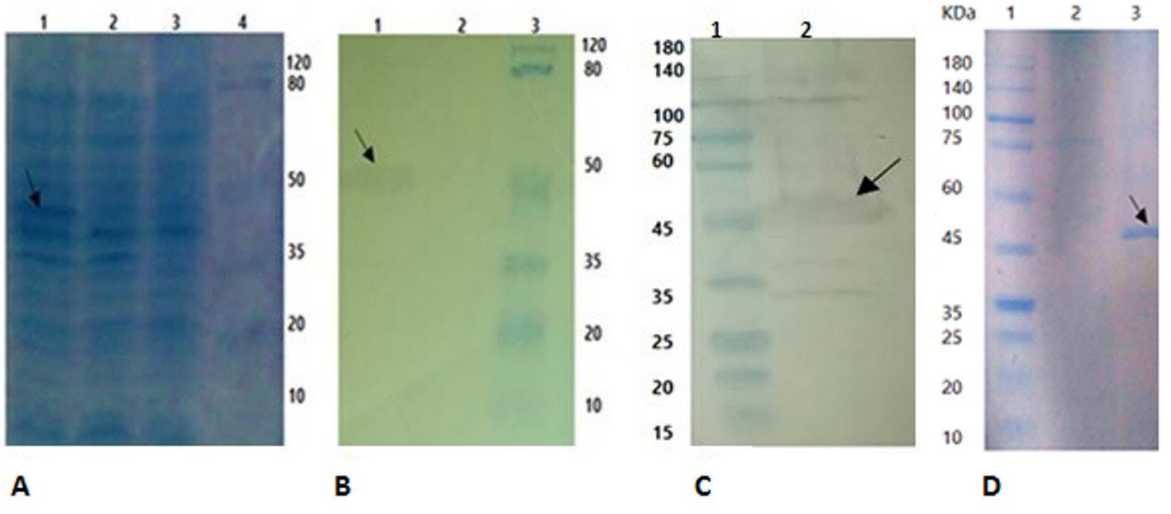

Fig. 3. SDS-PAGE and Western blot analysis of multi-epitope protein. (A) Expression analysis of rMEP. lane 1: induced BL21-rMEP ~ 45.5KDa; lane 2 un induced cells lysates, lane 3 control; lane 4: Standard protein marker. (B) Western blot analysis of rMEP using anti His tag antibody. lane1: rMEP with MW ~ 45.5KDa; Lane 2: un induced *E. coli* BL21 lysate. Lane 3: Standard protein marker. (C)Interaction of the rMEP and positive sera by Western blot analysis. Lane 1. Standard protein marker; lane 2. induced cells lysates. (D) Purification analysis of protein. lane 1: Standard protein marker; lane 2: Washed buffer; lane 3: Eluted protein ~ 45.5KDa

Table 2. The compared secondary structure of the purified protein predicted using GOR4 server with the secondary structure that revealed by Circular Dichroism

Secondary structure	Alpha-helix (%)	Beta-strand (%)	Random coil (%)
GOR4 predicted	41.93	13.01	45.06
Circular Dichroism	40.8	21.1	38.1

ELISA. Specific interaction between rMEP and total serum antibodies was measured by indirect ELISA in different dilutions of serum obtained from ICU patients and staff (Fig. 4A). A significant increase was observed in the reaction between serum dilution 1/100 and 0.5 µg rMEP multi-epitope in compare with control group (*E. coli* and PBS) (Fig. 4B).

DISCUSSION

Acinetobacter baumannii is difficult to manage due to its fast acquisition of antimicrobial resistance. Recently, WHO has introduced *A. baumannii* as a highly dangerous pathogen that threat human society. As well due to resistance to numerous antibiotics available considerate as bioterrorism organism (30). Vaccine production against it is a promising alternate and cost-effective strategy to prevent extreme- drug-resistant infections. However, no licensed vaccine is available yet in the market and numerous efforts have been made to introduce an actual vaccine against *A. baumannii*. In the past few years, reverse vaccinology is highly regarded for designing vaccines against various diseases, particularly infections (31, 32). Using immune-informatics tools for prediction of immunogenic antigens as well as epitope mapping of B- and T-cells with high accuracy can be effective in developing actual and safe vaccine against diseases. Several studies have shown that immunogenic B- and T-cell multi-epitope are able to provide protective immunity against various bacterial pathogens such as *S. aureus* (33, 34) and *Brucella abortus* (35). Therefore, in this research, we targeted a multi-epitope protein against *A. baumannii* strains. The *A. baumannii* genome encoded over 1,500 proteins that any of which alone or in combination can aid as a potential target for vaccine candidate (36). The Outer membrane protein A (38KDa) appears to be one of the highly immunogenic protein and an effective candidate for controlling *A. baumannii* infections (37, 38). In our previous study, high antibody titers against OmpA in the serum of recovering ICU patients and staff supports the role of this antigen in *A. baumannii* infections (27). The outer membrane protein A among different clinical isolates of *A. baumannii* is very conserved, while the lowest homology with human proteome. Many studies, however, have used OmpA to develop antibody protection in animal models (39, 40) but it is not sufficient by itself for protection against *A. baumannii* infection (31). Another very important antigen in Gram-negative bacteria is BAM (β-Barrel Assembly Machine) complex. The BAM complex (BamA, BamB, BamC, BamD and BamE) in *A. baumannii* is highly conserved due to its essential role in outer membrane protein assembly. More recently, Singh at al. have been reported significant immune-protective effects of BamA in a mouse pneumonia model which was created with clinically isolated lethal MDR *A. bumannii* (41). According to the present study, we designed a novel multi-epitope protein contains selected epitopes

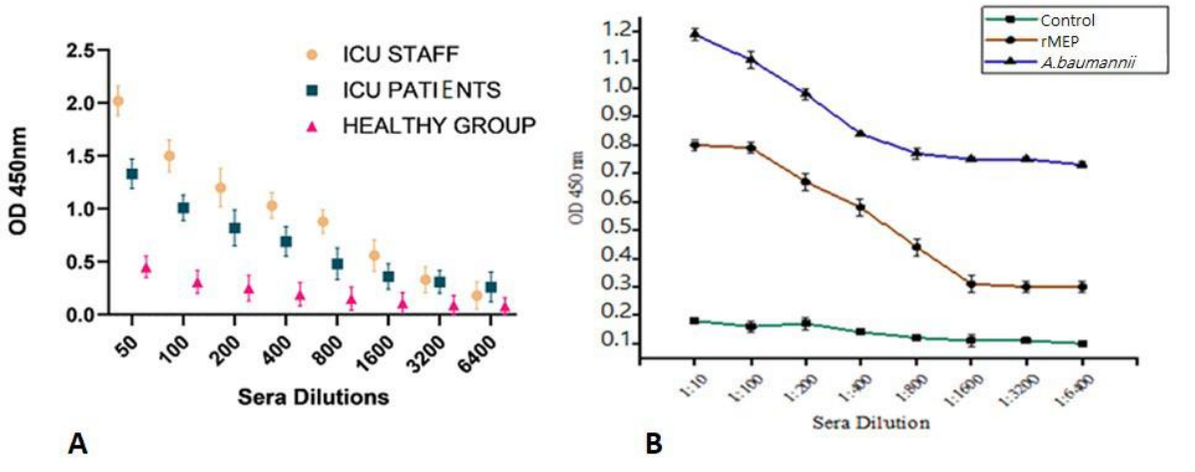

Fig. 4. ELISA analysis. A) The specific total antibody titer in different dilution of ICU staff, patients and healthy group sera in reaction with *A. baumannii* using ELISA assay. B) Comparative analysis of specific total antibody titer of sera in reaction with *A. baumannii*, recombinant multi- epitope protein(rMEP) and control group using ELISA assay. Data signifies Mean of the OD450 ± SD with P> 0.001.

of OmpA, BamA, BamB, BamC, BamD and BamE proteins. According to Ren et al. study designed multi-epitope vaccine with FilF and NucAB antigens could induce high levels of IgG antibodies and provide potent protection (88.9%) against lethal doses of *A. baumannii* (22). In further work by Singh et al. a multi-epitope subunit protein of BamA has shown high levels of protection in mice against *A. baumanni* (14).

To determine the B -cell and T -cell epitopes from selected virulence factors, we used the immune-informatics tools. Since humoral immune is crucial in protection against *A. baumannii* infection, multi-epitope protein was composed of linear B-cell epitopes and CD4+T-cell epitopes to develop an effective antigen. Furthermore, the MHC I and MHC II binding epitopes were designed specifically for the HLA-DRB1*11, 13, 15, 03, 04 alleles because of the high prevalence in the Iranian population (42). Consequently, based on antigenicity, sensitivity, toxicity, and solubility, 14 B- and T-cell epitopes were selected with the best scores. They were linked using a most widely flexible GGGGS linker so that no new epitopes were created. The small size of these amino acids provides flexibility, and allows for mobility of the connecting functional domains (43). The in silico results of this research showed that our multi-epitope protein named rMEP is stable, highly antigenic. To optimize protein expression in *E. coli*, some parameters, including Codon Adaptation Index (CAI), Codon Frequency Distribution (CFD) and GC content of genes were determined. The results confirm that desired protein structure can be fully expressed in the *E. coli* host. The three-dimensional structure of a protein plays a key role in protein binding and function. Therefore, the 3D structure of the rMEP was modeled using the I-TASSER server. Structural quality and stability of the predicted model were evaluated using Ramachandran map, ProSA Z-score and ERRAT quality factor. The high structural quality of the protein was verified by the obtained results. As mentioned previously, recombinant protein was produced in the form of the inclusion body in the *E. coli* BL21 (DE3). Protein denaturation was performed using urea 8M, to obtain soluble and active form of protein. In this study, the purified protein was refolding to the active form by stepwise urea removal using Amicon-30KDa filter. Circular Dichroism spectrophotometry analysis was performed to evaluate the effect of purification conditions on recombinant protein refolding. The results showed that after purification, the recombinant protein retained its structure almost correctly, although the percentage of secondary structures changed slightly. Finally the multi epitope DNA sequence with LC521880 accession number was released in the DDBJ data bank.

On the other hand, antibodies, as primary mediators of the immune system, perform various functions to remove antigens and control infections. Antibodies, opsonizing the pathogens and increase phagocytosis activity of macrophages also activate the complement system, leading to lysis of bacteria. In this research the results of ELISA showed high antibody titers in ICU staff and recovering patient's sera that is supports the role of antibody in *A. baumannii* infections could interact with selected fragments in rMEP. In previous study we showed that predicted peptides in OmpA protein from *A. baumannii* could be the efficient epitopes as part of novel vaccine design in animal study (44). Also, to confirm the efficiency of peptides in vaccine design, researchers have been able to use peptides similar to other bacterial components for this purpose. For example, mimicking lipopolysaccharide as a potential vaccine candidate against *Vibrio cholerae* serogroup O1 (45). Therefore, obtaining effective peptides to stimulate the immune system is one of the most cost-effective and safe methods for vaccine design.

In conclusion, we designed and evaluated a novel protein consisting of high score B and T cell epitopes from crucial virulence factors OmpA, Bam A, Bam B, BamC, Bam D, Bam E belong to *A. baumannii*. Also the sera of the ICU staff and recovering patients with a history of *A. baumannii* infections have been able to interact with this protein.

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